INCORPORATION OF ANALGESICS INTO RODENT EMBRYO TRANSFER PROTOCOLS: ASSESSING THE EFFECTS ON REPRODUCTIVE OUTCOMES

A Thesis

by

HEATHER ANN BURCKHARDT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2006

Major Subject: Laboratory Animal Medicine

INCORPORATION OF ANALGESICS INTO RODENT EMBRYO TRANSFER PROTOCOLS: ASSESSING THE EFFECTS ON REPRODUCTIVE OUTCOMES

A Thesis

by

HEATHER ANN BURCKHARDT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Melanie M. Ihrig
Committee Members,	Ann B. Kier
	Jane C. Welsh
Head of Department,	Gerald R. Bratton

December 2006

Major Subject: Laboratory Animal Medicine

ABSTRACT

Incorporation of Analgesics into Rodent Embryo Transfer Protocols:
Assessing the Effects on Reproductive Outcomes. (December 2006)
Heather Ann Burckhardt, B.S., Texas A&M University
Chair of Advisory Committee: Dr. Melanie M. Ihrig

Surgical embryo transfer in rodents is a common procedure in today's research laboratory, although little is known of the effect analgesics may have on not only the recipient female but also the embryos. Two perioperative analgesics, ketoprofen and buprenorphine, were evaluated against a saline control in terms of number of pups born, number of pups weaned, and whether or not a litter was born. Both a uterine approach and an oviduct approach were evaluated. Post-surgical behavior was compared among the three surgical animals in each group, and between the non-surgical analgesic control and its surgical counterpart. Results indicated that ketoprofen and buprenorphine have no effect on the number of pups born, weaned, or litters born when compared to a saline control. Significant differences were found between the non-surgical analgesic control and its surgical counterpart in two behavioral categories; once for ketoprofen (behavior) and once for buprenorphine (physical condition). No other differences were found.

ACKNOWLEDGEMENTS

First and foremost I wish to acknowledge my committee chair and principle investigator, Dr. Melanie Ihrig. Without her, there would not have been a project, nor this rich learning experience. For your guidance, encouragement, and example, I thank you.

I would like to express my gratitude to my committee members, Dr. Ann Kier and Dr. Jane Welsh. Thank you both for your insights and comments that helped when I had blocks, your questions that made me think, and your conversations that let me see the person behind the title.

Stacy Galavez, the surgeon for this project, and Jean Kovar, the colony manager, are deserving of more than I can express here. I know both of you ended up in way over your heads, but you both bore the responsibilities with such grace. Without the two of you, this project would have never been finished, and for that you have my eternal thanks.

The Animal Health team and caretakers at CMP deserve thanks for taking care of our animals. Their vigilance and dedication to duty was essential to this project, and so I thank you.

To my family and my friends, who offered shoulders to cry on, things to beat up, and stuff to do when I needed a break, perhaps goes the biggest thanks of all. I would not have kept my sanity without all of you.

And finally, first, last, and always, my friend, my confidant, my partner: To Travis – thanks for the fruit hat.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

Page

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 HISTORY

The procedure of transferring embryos from one female to a second recipient female is one that has been around for at least a century. Walter Heape performed the first successful experiment in 1890. In this groundbreaking procedure, he took fertilized eggs from an Angora rabbit and placed them within the fallopian tube of a bred Belgian doe. The Belgian doe eventually gave birth to six kits, two of which were "undoubted Angoras" in Heape's own words. This original experiment was conceived to determine what effect a recipient foster-mother would have on the implanted embryos. Its second goal was to determine what effect the presence of foreign embryos in the uterus would have on the native offspring of the recipient female (18).

Since that particular experiment, there have been many other noteworthy and successful attempts using the mouse as the experimental animal. The first such published successful attempt was by Bittner and Little in 1937. This was followed by subsequent publications by Fekete and Little in 1942, Fekete in 1947, Beatty in 1951, Runner in 1951, Runner and Palm in 1953, and Boot and Muehlbock in 1953. It was Runner and Palm in 1953 who discovered and published the technique used today of inducing ovulation in the mice via the injection of hormones (18, 1, 19).

The next major landmark in the history of embryo transfer occurred in 1956, with a paper written by Anne McLaren and Donald Mitchie entitled "Studies on the Transfer of Fertilized Mouse Eggs to Uterine Foster-Mothers." This paper, published by the Journal of Experimental Biology, outlined a massive experimental undertaking in which many

---

This thesis follows the style of Comparative Medicine.

questions that had arisen since the first transfer in 1890 were addressed. Some of the areas of interest included the effect of foreign ova on the recipient female herself; the timing of coitus between the donors and the recipients; the possibility of an "ideal" number or a "ceiling" to the number of eggs that could be implanted per uterine horn; the effect the foreign embryos had on the native ones; the effect surgical trauma had on the recipient female; and the sexual maturity of the donors (18).

Through the work of McLaren and Michie it was determined that immature, induced-ovulation female donors at 3.5 days post-coitus (dpc) at surgery and recipients at 2.5 dpc yielded the most successful results (18). This particular combination gave nearly twice the yield of live, full-term young as compared to the other time-point combinations. These findings have been incorporated into embryo transfer protocols and are still used today (7, 1, 28, 12, 16, 17). Recipient mice are mated a full day after the donors to ensure the proper uterine environment for the embryos.

McLaren and Michie also studied the effect of surgery on the recipient mothers. They found no statistically significant difference between the groups of non-surgical pregnant control mice and the embryo transfer recipient mice that became pregnant (18). In "dummy" transfers, where saline without embryos was injected into the uterine horn of a pregnant recipient, the number of implanted embryos suffered. It was thought that the saline flushed away the native embryos in the injected horn, resulting in a smaller number of implants. Later experiments supported this view, as when the saline was loaded with foreign embryos the implantation number actually increased in the injected horn as compared to the uninjected one (18).

Their findings also took into account the effects of “traumatic” vs. “non-traumatic” operations, two subjective categories determined by the investigators themselves (18). Recipients at 3.5 dpc had an overall greater resorption rate, and that rate increased when subjected to a “traumatic” surgery. In contrast, the 2.5 dpc recipients had the same rate of absorption whether the surgery was classified as traumatic or not. However, severe trauma can result in post-implantational mortality even in 2.5 dpc recipients (18, 16).

In pregnant recipient females, there is competition between “native” and “alien” embryos for implantation in the uterus (18). This would explain the greater number of implanted foreign embryos in the injected horn: at 3.5 dpc, the foreign embryos are further along in their development and ready to implant in a primed uterus. The native embryos, only at 2.5 dpc, are not ready to implant and are shunted aside (18). This series of experiments was repeated with pseudopregnant recipient females; in these operations nearly all of the eggs injected implanted, suggesting that there is competition between the native and alien embryos. Most embryo transfers done today utilize a pseudopregnant female to ensure that as many injected embryos as possible implant (7, 28, 12, 16). A pseudopregnant female is one who has been bred to a vasectomized male. The male does not have the ability to impregnate the female, but due to the act of mating her body behaves as though she is pregnant. This prepares the uterus to be more receptive to the injected embryos.

A question addressed in these experiments was whether or not there was a maximum number of implants a uterine horn could cultivate. Although there did not seem to be a maximum per horn, there was a ceiling on the total number of implants. As long as the

total number of implants for both horns did not exceed this number, it did not matter how many implanted per side (18). Researchers involved in transgenic mouse production inject many manipulated embryos into each uterine horn to maximize the number that implant and survive (16). If too many implant, however, the rate of absorption in the uterine horn increases. Trial and error must be used to determine the optimal number between introducing as many embryos as possible, and injecting so many that most are absorbed or the entire innoculum is lost (18).

McLaren and Michie undertook this experiment with far-reaching goals in mind. They saw the potential this procedure had in research and practical applications in livestock and even humans as well. In the introduction to their published work, in fact, they cite the "range of problems in genetics' embryology, reproductive physiology, immunology and cancer research" that could be studied and perhaps even solved with the use of this new technique (18). Applications to livestock were mentioned as well, describing a situation in which a sexually immature yet valuable female could be induced to ovulate, the eggs collected and fertilized *in vitro*, then either frozen for later use or transferred immediately into a sexually mature recipient female. The same idea has been in practice for many years, in which artificial insemination is used to propagate the genetic superiority of a male (1, 6). This would be a similar procedure, in which the genetics of a female could be passed on without even waiting for the female to become mature enough to bear her own young.

1.2 TARGETED MUTANT MOUSE PROJECT

Little has changed in embryo transfer from the original research undertaken almost fifty years ago. Laboratory manuals published on the subject cite the "extensive work" by McLaren and Mitchie as the basis for their surgical technique (14, 21). The same technique developed in 1956 has been used in a variety of procedures, from producing germ-free mice, to clearing a colony of a persistent mouse hepatitis virus (MHV) infection, to creating new lines of transgenic animals, to reinstating a line after cryopreservation (4, 13, 23, 25, 2, 31, 20).

In an interview in 1986, on the 30th anniversary of her ground-breaking experiment, Ann McLaren stated that "there are certain procedures, such as injection of genes or nuclei into eggs, that in my view are too remote … to be … discussed at the present time (5)." Just one year after McLaren's pronouncement, a researcher by the name of Mario R. Capecchi was experimenting with that technique (3).

In his paper, Capecchi talks of the vast amount of work spent on analyzing countless numbers of mutations when it was unknown "which mutations [were] worthy of detailed characterization (3)." This premise has changed, Capecchi says, because the researcher no longer has to wait for random mutagenesis: "Through gene targeting, the potential now exists to generate mice of any desired genotype (3)." This method has far-reaching potential, from removing the functionality of a gene, to causing it to overexpress, to introducing genes from an entirely new species into a genome (8, 28). Embryo transfer is of particular import to this endeavor. It is through this procedure that the genetically

modified embryos are placed within a viable uterus in the hopes that they will develop into animals with the desired modification.

Generally, there are two types of genetically modified mice. The first is what is known as a transgenic mouse. The transgenic mouse has a *transgene*, a gene taken from the genome of another organism and introduced into its own. The purified DNA containing the transgene is injected into the pronucleus of a fertilized 1-cell egg, called a zygote. This insertion results in stable germline transmission of the microinjected DNA around 1% of the time. The egg is then transferred into a pseudopregnant recipient female. The transgene is integrated randomly and transmits as a "Mendelian trait", which is one that segregates in accordance with the laws of genetics set forth by Gregor Mendel (29). Germ-line founder mice, those in whom the transgene integrated into their germ cells, are bred to produce the transgenic line (29, 28). The advantage of this method is that there are no size limitations on the transgene (28). The disadvantage, as stated before, is that the insertion integrates randomly into the genome: the researcher has no control over where it goes (29, 28). This random insertion may occur in the midst of a functionally important gene, thereby disrupting it and producing an unexpected and possibly undesirable phenotype.

The second type of genetically modified mouse is the targeted mutant, commonly known as the "knockout" mouse (28). In these animals, "specific endogenous genes have been disrupted and rendered inactive by insertion of nonfunctional exogenous DNA sequences (28)." These mice are created using embryonic stem (ES) cells derived from

mouse blastocysts. These cells were first cultured in 1981 by Drs. M. Evans and G. Martin (28). ES cells are pluripotent, meaning they can develop into any type of tissue.

In brief, the procedure for creating genetically modified ES cells follows. Blastocysts from superovulated donor mice are harvested and the inner cell mass is removed and placed in culture. A DNA construct is then introduced to the ES cell culture. "Typically, a targeting vector is designed" that utilizes a "positive selectable marker such as the *neo'* gene" which confers resistance to the drug neomycin (28). Since the *neo'* gene is placed in the middle of the construct, integration will confer the drug resistance. When neomycin is introduced into the media, the cells which have not integrated the construct are killed. A second selective method is one that involves a negative selective factor, such as one that makes the cells sensitive to the drug gancyclovir, placed at the end of the construct. If the construct is integrated correctly, the part containing the sensitivity will be eliminated during homologous recombination. If the construct is integrated randomly, the selection factor will be maintained and the cells will die when gancyclovir is introduced to the culture (28).

Targeted ES cells are microinjected into donor mouse blastocysts then transferred into the uterine horns of a pseudopregnant recipient female, which is a female that has been bred to a vasectomized male so as to prepare the uterus to accept embryos. Resultant offspring are called "chimeras" because they are part host embryo-derived and part ES cell-derived (28). If the strains that provided the ES cells and host cells are of differing colors, the chimeric coat pattern can be easily identified. "Typically, chimeras are generated from strain 129/Sv (agouti coat color) ES cells and strain C57BL/6 (non-

agouti, black coat color) host blastocysts (28)." To test for germline transmission, the chimeras are mated to C57BL/6 mice. If the offspring are agouti, they developed from the 129/Sv ES germ cells and the chimeric parent had germline integration. If they are black, the offspring developed from the BL/6 host blastocyst and the chimeric parent does not contain the construct in their germ cells (28).

The selection of mouse strains for use in genetic manipulation is crucial. In all cases, the eggs must be hardy enough to withstand the manipulation procedure itself. Also, if a large number of eggs are required then a donor strain must be selected that responds well to superovulation (27). Lee M. Silver's book Mouse Genetics, based on strain research at NIH, looks closely at these and other characteristics (27).

The C57BL/6 mouse responds well to superovulation. 3 week-old females can be induced to produce anywhere from 40-60 eggs with appropriately timed injections of follicle stimulating hormone and human chorionic gonadotropin (27). It is also the "most widely used inbred strain (15)." It has had its entire genome mapped, making it an excellent candidate for transgenic production. It is also used as a general purpose model and as a background strain for "generation of congenics carrying both spontaneous and induced mutations (15)." It was for these reasons that this strain was selected as the embryo donor for this project.

1.3 CRYOPRESERVATION

With the wide-spread use of genetic manipulation to create novel strains of mice for study, new opportunities emerged for research. However, with those opportunities

arose an unforeseen difficulty: the increased burden on facilities due to the number of animals needing to be housed. The answer to this difficulty was cryopreservation.

This technique was first performed successfully in 1972 on mouse embryos. Since then, the process has been modified in 1977 to preserve mouse oocytes and in 1983 to preserve human embryos. Today, cryopreservation is a common laboratory practice, and mouse embryos of all preimplantation stages can be successfully frozen (21).

Cryopreservation is an important and vital procedure in any laboratory that engages in genetic manipulation of embryos for several reasons. It "reduces maintenance costs and safeguards valuable mouse lines against loss (21)." The cause of these losses can be attributed to anything, from infection and disease to breeding failure and genetic drift or contamination. The loss of the research animals can also be indicative of husbandry problems, such as flooded cages, inaccessibility to food, or similar conditions.

This technique is also extremely valuable when rederiving lines following infection. Although conventional or infected strains can be introduced into a specific-pathogen-free (SPF) colony by hysterectomy of pregnant females just before giving birth, there is still a concern that certain viral and bacterial infections can be transmitted vertically. The use of cryopreservation and embryo transfer "has the advantage of avoiding postimplantation, vertically transmitted infections (21)." Cryopreservation and embryo transfer have been used together to successfully eliminate mouse hepatitis virus (MHV) and Sendai (parainfluenza type 1) virus from colonies (21).

There are a number of factors to consider when utilizing cryopreservation. Although all preimplantation stages have been successfully preserved, mouse embryos at the eight-cell- and morula-stage are preferred due to their robust nature. Unlike one- and two-cell-stage embryos, they tolerate handling and cryopreservation well. Upon thawing, embryos can be transferred immediately into a pseudopregnant recipient female, or they may be cultured in the incubator to determine viability (21).

1.4 PAIN

One of the many subjects addressed by McLaren and Michie in their original research was the effect of the surgical procedure on the recipient mice (18). Would the trauma caused by the operation render the uterus incapable of nourishing the embryos and bringing them to term? Although their findings suggested that the procedure has no adverse effect on the ability of the female to carry the embryos to term, post-operative recovery and pain is a concern in any surgical procedure.

The embryo transfer procedure is classified as major surgery, according to the Guide for the Care and Use of Laboratory Animals, because it penetrates and exposes a body cavity (22). An incision is made in the dorsal body wall over the ovarian fat pad and the uterine horn is exposed through this incision. Although the animal is unaware of the pain during the surgery, it is likely that there is discomfort during and perhaps even after recovery.

The debate on whether animals feel pain as humans experience it is one that has raged for centuries. One end of the debate can be neatly summarized by Descartes, who was quoted as saying, “The greatest of all the prejudices we have retained from our

infancy is that of believing that the beasts think (9)." He believed, as do a great many others, that since animals lack the reasoning ability of humans they do not have the perception of pain that humans do. When confronted with avoidance behaviors and other reactions in response to stimuli that would cause pain in people, Descartes merely responded that these were simply reflexes: the "response of automatons (9)."

The other side of the debate is often represented by a quote from Jeremy Bentham, an 18th century utilitarian philosopher. He is quoted as saying, "The question is not can they reason? Nor can they talk? But can they suffer (9)?" *Suffering* is defined by the Handbook of Veterinary Pain Management as "the endurance of or submission to physical or mental affliction, pain, or loss (10)." The dog with his abdomen held tense and tight; the cat who curls up in one corner of its cage in order to avoid moving; the rat who twitches and jumps in its uneasy sleep, waking every few moments to stretch and rearrange itself into a possibly more comfortable position; all of these are specific examples of animals in discomfort – animals who are submitting to their pain (9).

Aside from the important ethical reasons, there are also scientific reasons for minimizing pain in research animals. Animals in a stressful situation, such as being restrained or put into a new environment, will immediately produce a stress-response in their bodies (10). The physiologic responses to stress, such as increased heart rate and breathing rate and the release of epinephrine, actually serve to exacerbate any discomfort the animal is feeling. Such responses may be physically damaging to the animal, and may also serve to confound research data (9).

In order to properly treat pain, it is important to understand how the body interprets and responds to this stimulus. Physiologists have determined that pain is a protective mechanism that serves to warn the body that tissue damage is occurring or is about to occur. The body categorizes pain into three separate groupings, each one represented by a different type of nociceptor, or pain receptor. The three receptors are mechanical, thermal, and polymodal. Mechanical nociceptors respond to pain that results from such things as cutting, crushing, or pinching. Thermal nociceptors respond to extreme temperatures, most notably heat. Polymodal receptors respond equally to many different types of damaging stimuli. Nociceptors, unlike other nerve receptors in the body, are simply naked nerve tissue with no specialized receptor structures. Also unlike other nerve receptors in the body, they do not adapt to repeated or sustained stimulation. They do, however, react more strongly whenever prostaglandins are present. Prostaglandins, which are fatty acid derivatives that are released whenever tissue is damaged, lower the activation threshold of the nociceptors (10, 26).

Whenever a painful stimulus is experienced, the impulse travels from the point of contact to the central nervous system via one of two different pathways. Pain signals from mechanical or thermal nociceptors travel along small myelinated A-delta fibers at incredibly high speeds. This pathway is termed the fast pain pathway. It is experienced as a sharp, prickling sensation, easily localized. It is the first type of pain to occur. The second type of pain to occur is stimulated by the polymodal nociceptors. This message travels much slower to the CNS via small unmyelinated C fibers. This is what produces the dull, aching, burning sensation that follows the sharp pain immediately felt upon

injury. This type of pain is harder to pinpoint and lasts much longer than the fast pain (10, 26).

As the pain fibers synapse with second-order interneurons in the dorsal horn of the spinal cord, two different neurotransmitters are released. Substance P activates ascending pathways that relay the pain signal to higher levels for further processing. These pathways eventually end in the somatosensory cortex, the thalamus, and the reticular formation. It is this part of the pain pathway that is unclear. It is thought, however, that the reticular formation increases the alertness associated with the painful encounter. Connections between this and the hypothalamus and limbic system elicit the emotional and behavioral responses that accompany the painful experience (10, 26).

Glutamate, the second neurotransmitter released by the afferent pain fibers, is a major excitatory neurotransmitter. Glutamate binding to two different plasma-membrane receptors on the dorsal horn neurons serves two different purposes. First, when it binds to AMPA receptors it generates an action potential in the dorsal horn cell. These action potentials serve to carry the pain message to higher nerve centers. Secondly, when it binds to NMDA receptors it allows calcium ions to enter the dorsal horn cell, which makes the neuron more excitable than usual. This results in the exaggerated hypersensitivity of an injured area (10, 26).

1.5 ANALGESICS

There are four classes of analgesic drugs: opioids, nonsteroidal anti-inflammatory drugs (NSAIDs), alpha-2 agonists, and local anesthetics (10). This paper will focus on the opioids and the NSAIDs, as the drugs used in this study belong to those two classes.

As defined by the Handbook of Veterinary Pain Management, opioids are “any one of a growing number of natural or synthetic compounds that produce morphine-like effects by acting on opioid receptors (10),” the desired effect being analgesia. Opioid receptors can be found on the endings of afferent pain fibers that release Substance P. Opioids act by blocking the release of Substance P and thereby blocking the pain signal from entering the higher levels (10).

Opioids are classified into three groups: 1) antagonists, 2) agonists, and 3) agonist-antagonists and partial agonists. Opioid antagonists do not produce analgesia and are used to either block or reverse the effects of opioid agonists. Opioid agonists work at the opioid receptors by binding and producing the analgesic effects. Agonist-antagonists and partial-agonists produce the analgesic effects of the agonists but not as strongly and are generally less toxic. Studies have shown them useful for helping heroin addicts overcome withdrawal symptoms (9).

Opioid receptors have three different subtypes, and each opioid shows a certain affinity and action for each of the subtypes. Mu receptors are responsible for supraspinal, spinal, and peripheral analgesia. Opioids that act at this receptor give a minimal to mild sedation. Kappa receptors are responsible for supraspinal and peripheral analgesia and possibly spinal analgesia as well. Drugs that work at this receptor give minimal sedation. Delta receptors give supraspinal, spinal, and peripheral analgesia and minimal sedation. A fourth receptor, sigma, was once included but is now recognized as not fitting the required criteria (9, 10).

Nonsteroidal Anti-inflammatory Drugs, commonly known as NSAIDs, are perhaps the most popular class of analgesics in use in small animal veterinary medicine, according to the Handbook of Veterinary Pain Management. NSAIDs produce analgesia by inhibiting arachidonate cyclooxygenase synthase (COX), which in turn produces prostaglandins. Prostaglandins lower the activation threshold of nociceptors which makes them more responsive to less stimuli (9, 10).

There are two different types of COX. COX-1 is responsible for maintaining tissue homeostasis and is involved in cell signaling. COX-2 is induced in inflammatory cells and is responsible for the production of inflammatory mediators. The inhibition of COX-1 is thought to be responsible for the acute and chronic toxicities produced by some NSAIDs. Most NSAIDs available today inhibit both isoforms of COX, so it is important to be aware of proper dosages (9, 10).

For this project, one drug from each of these two categories, buprenorphine and ketoprofen, was selected on the basis of Paul Flecknell's research into rodents and pain. Buprenorphine is a very potent opioid partial-agonist at the mu receptors. It has an extremely high affinity for these receptors, although it is very slow to bind with them. This is reflected in the slow onset of action displayed by this drug, which can be anywhere from 30 minutes to an hour. Buprenorphine tends to stay in the body for long periods of time, with a half-life in dogs of around 42 hours. However, it does not have a long duration of action in the dog, which can last anywhere from 4 to 8 hours. There have not been many clinical trials of the use of buprenorphine or other opioids in small rodents. Buprenorphine has the longest duration of action of the opioids, which makes it

a clear choice for use in small rodents, as their small body size and fast metabolism enables them to clear drugs from their system much faster than a dog. The best time to dose a rodent remains unknown, however. The long onset of action leads some to believe that it is best to dose pre-operatively, while some studies undertaken show that a single dose of buprenorphine either intraoperatively or post-operatively does well for controlling surgical pain. One thing to remember when using this drug, however, is that at higher or more frequent dosages the antagonist action takes over, actually rendering the drug useless at providing analgesia. Smaller dosages appear to work best at providing analgesia (9).

Ketoprofen is licensed for use in a wide variety of animals, such as the dog, cat, horse, and cow. It is a very potent non-selective inhibitor of COX and displays both analgesic and anti-inflammatory properties. Post-operative use in dogs and cats to provide pain relief has been reported to be very effective, although there is concern with toxicity when given over prolonged periods. As with the opioids, there is little clinical experience in giving ketoprofen to small rodents. However, its use is becoming more widespread and no evidence of adverse reactions has yet been reported. It is advisable to dose post-operatively to give the greatest benefit of analgesia possible; some reports have stated that a single dose can provide up to 24 hours of relief (9).

Many researchers are reluctant to use analgesics in their animals for multiple reasons. Analgesics may not have been used when collecting previous data, thereby calling into question the comparative validity of results. Some investigators do not wish

to add another variable to their work, especially since few studies have been done on the effects analgesics may have.

For this study, three surgical groups of mice were used to determine the effects of analgesics on embryo transfer procedures. For each experiment, a set number of embryos was injected into each of three mice, which were dosed either with ketoprofen, buprenorphine, or saline as a control. Mice were evaluated post-operatively for pain behaviors. Each mouse was observed and the number of pups born and the number of pups that survived until weaning was recorded and measured against the number of embryos injected.

This research project was undertaken to provide investigators more data regarding the effects of analgesic administration on reproductive efficiency in mice. The increased use of analgesics is heavily encouraged, yet there is little information detailing the effects the administration of such drugs may have. This is of critical import in embryo transfer, especially considering the number of laboratories using this procedure across the country. Embryo transfer has a wide range of applications, from rederivation of a genetic line after a disease outbreak, reinstating a line following cryopreservation, to production of an entirely new transgenic or knock-out animal for study. Efficiency of transfer in such a situation is very important, especially when taking into account the number of embryos that must be manipulated and transferred to ensure founders are produced. Two separate types of analgesics were studied to compare the effects on reproductive efficiency.

2. MATERIALS AND METHODS

2.1 ANIMALS

The procedures enumerated here were done with the approval of the Texas A&M University Laboratory Animal Care Committee, following Institutional Animal Care and Use guidelines. Female and male C57BL/6 mice from NCI, female Swiss Webster mice from Harlan and vasectomized Swiss Webster and ICR male mice from Harlan were selected for this project. All animals were housed at Texas A&M University's Laboratory Animal Resources and Research (LARR) facility, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), International. All animals were housed and cared for according to the Guide for the Care and Use of Laboratory animals. Females were housed five to a cage and separated by strain, whereas the males were singly-housed. All were housed in standard clear polycarbonate rodent cages (standard polycarbonate mouse cage, 17"x8.5"x6"; Allentown Caging Co., Allentown, NJ) with pine bedding (PWI Brand; PRO-CHIP 8/16, Aspen Hardwood Sawdust; P.W.I. Industries, Inc.) and kept on a 12L:12D light cycle (lights on at 0700 and off at 1900). After 18 experimental groups had undergone treatment, the light cycle was changed to a 14L:10D (lights on at 0600 and off at 2000) for the remaining 35 groups. Humidity was kept at around 60% and the temperature held at a constant 70^{o}F, +/- 3 degrees. Animals had free access to a 4% standard laboratory chow (Harlan Teklad Laboratory Diets; Teklad Rodent Diet (W); 8604). After 18 experimental groups had undergone treatment, the females in the study

were changed to a 9% fat diet (Lab Diet: Mouse Diet 9F #5020). Tap water was available *ad libitum*.

2.2 SUPEROVULATION

C57BL/6 embryo donor females were injected intraperitonealy (i.p.) with 0.1 ml (5 I.U.) of PMSG (gonadotropin; Pregnant Mare Serum; G-4877; Sigma-Aldrich Co) followed 48 hours later with an i.p. injection of 0.1 ml (5 I.U.) hCG (chorionic gonadotropin; CG-5; Sigma-Aldrich Co). Upon arrival, PMSG was diluted to 200 I.U. (1ml) aliquots and hCG diluted to 500 I.U. (1ml) aliquots. Three mls of saline was added to the PMSG so that 4ml equaled 200 I.U. Once this solution had been thoroughly mixed, 0.1ml (5 I.U.) was drawn up into tuberculin syringes and frozen at 4^0C until needed. To the hCG was added 9ml of saline so that 10ml equaled 500 I.U. After agitating the solution to ensure even distribution of the hormone in the saline, 0.1ml (5 I.U.) were drawn up into tuberculin syringes and frozen at 4^0C until needed. Following hCG administration, mice were immediately placed with intact males of the same strain and checked the following morning for copulatory plugs. Plugged females were considered to be at 0.5 day pregnancy. Plugging success for the males was recorded on their cage cards. All females were returned to their home cages regardless of whether they plugged or not. Recipient females were placed with vasectomized males in a 1:2 breeding, two females placed in a single male's cage, that same afternoon. Mice were checked the following morning for copulatory plugs, which was recorded as 0.5 day pseudo-pregnancy. Plugging success for the males was recorded on their cage card. Plugged females were placed in a separate cage while unplugged females were returned

to their home cages. Two unplugged females were selected as non-surgical controls. For a uterine transfer, surgery was scheduled so the embryos would be 3.5 days at collection and the recipients would be 2.5 days post-coitus. For an oviduct transfer, surgery was scheduled so that the embryos would be 2.5 days at collection and the recipients would be 0.5 days post-coitus.

2.3 EMBRYO COLLECTION

The evening before a uterine transfer surgery, a four-well plate was prepared with M16 solution (M7292; embryo tested; Sigma-Aldrich Co) and mineral oil (M8410-500ML; embryo culture tested; Sigma-Aldrich Co) and placed in a water-jacketed CO_2 incubator (Nuair US Autoflow CO_2 Water-Jacketed Incubator, Model NU-4750; 37^oC, 5.7% CO_2). On the morning of surgery, M2 solution (M7167; embryo tested; Sigma-Aldrich Co.) was removed from the refrigerator and placed in a petri dish one half hour before collection to warm it to room temperature. Donor females were sacrificed via cervical dislocation around 0700. The entire uterus was removed and placed in a small petri dish of M2 solution. Removal of the uterus from the five females took approximately 10 minutes. The uterus was flushed with a 3ml syringe filled with the room-temperature M2 solution and a 27G or 30G needle. Each uterus was flushed into its own petri dish so that embryo yield per animal could be recorded. Flushing took approximately 10-15 minutes. After flushing, embryos were counted and graded to determine which would be transferred.

The grading system was a scale from 1 to 4. Embryos of “excellent” or “good” quality were enumerated as 1 (Figure 1). These embryos had an inner cell mass as well as a proliferative trophectoderm layer of cells. “Fair” embryos were called 2 and also had an inner cell mass. Most of the trophectoderm layer of cells were actively reproducing. Characteristics of a grade 3 or “poor” embryo were a barely visible inner cell mass as well as less than half of the trophectoderm layer of cells actively proliferating. Vacuoles were present nearly all of the time. “Dead or degenerating” embryos were called 4 and displayed no inner cell mass, and had no proliferation of the trophectoderm layer of cells as well as a completely vacuolated cavity. These embryos were also frequently dark when viewed as compared to embryos of other grades, a visual signal that they were dead or dying (Figure 2). Only embryos of 1 or 2 quality were used in surgical procedures. Embryos of 3 or 4 quality were placed back into the incubator to be used for non-survival surgical practice.

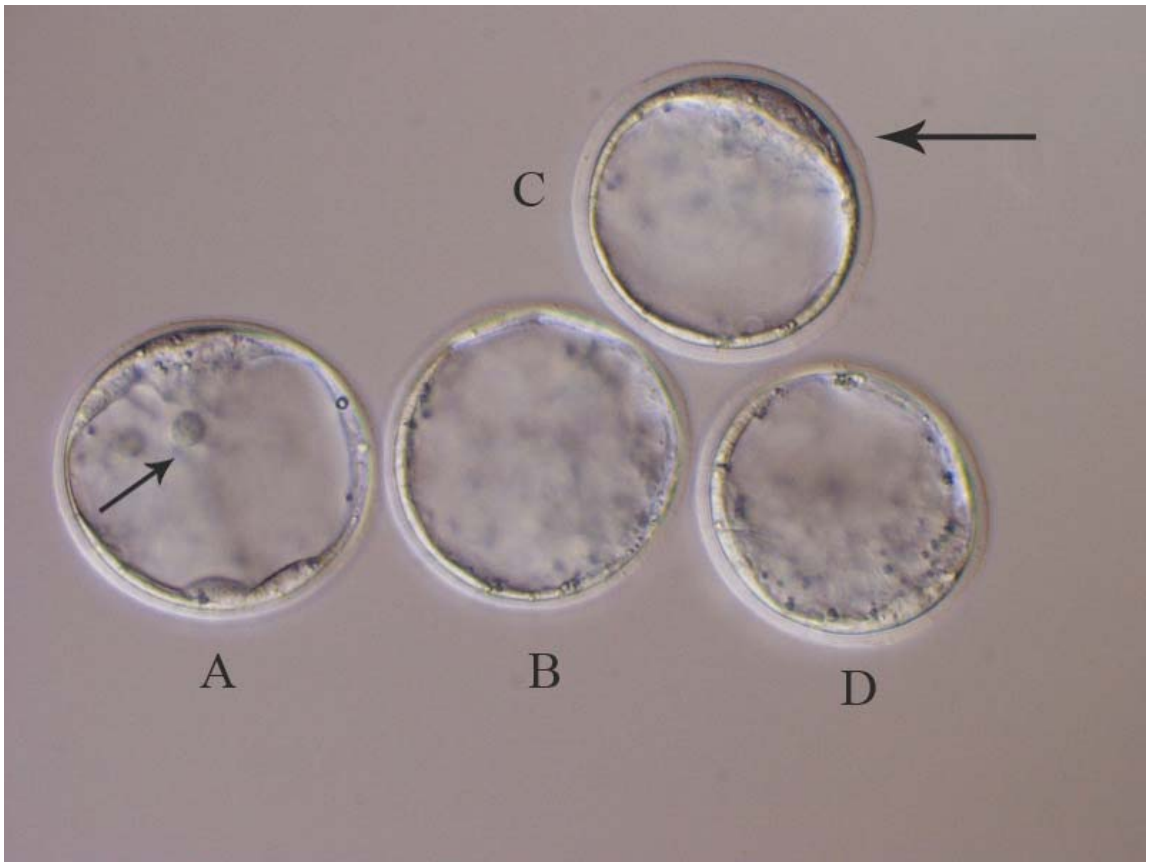

Figure 1. Embryos, Grade 1 and 2. Embryo (A) is of grade 2 due to the presence of foreign bodies (indicated with arrow). Embryos (B) and (D) are of grade 1 and are expanded blastocysts. Embryo (C) is actively expanding. The arrow indicates the proliferating trophectoderm layer and the last remaining vestige of the inner cell mass.

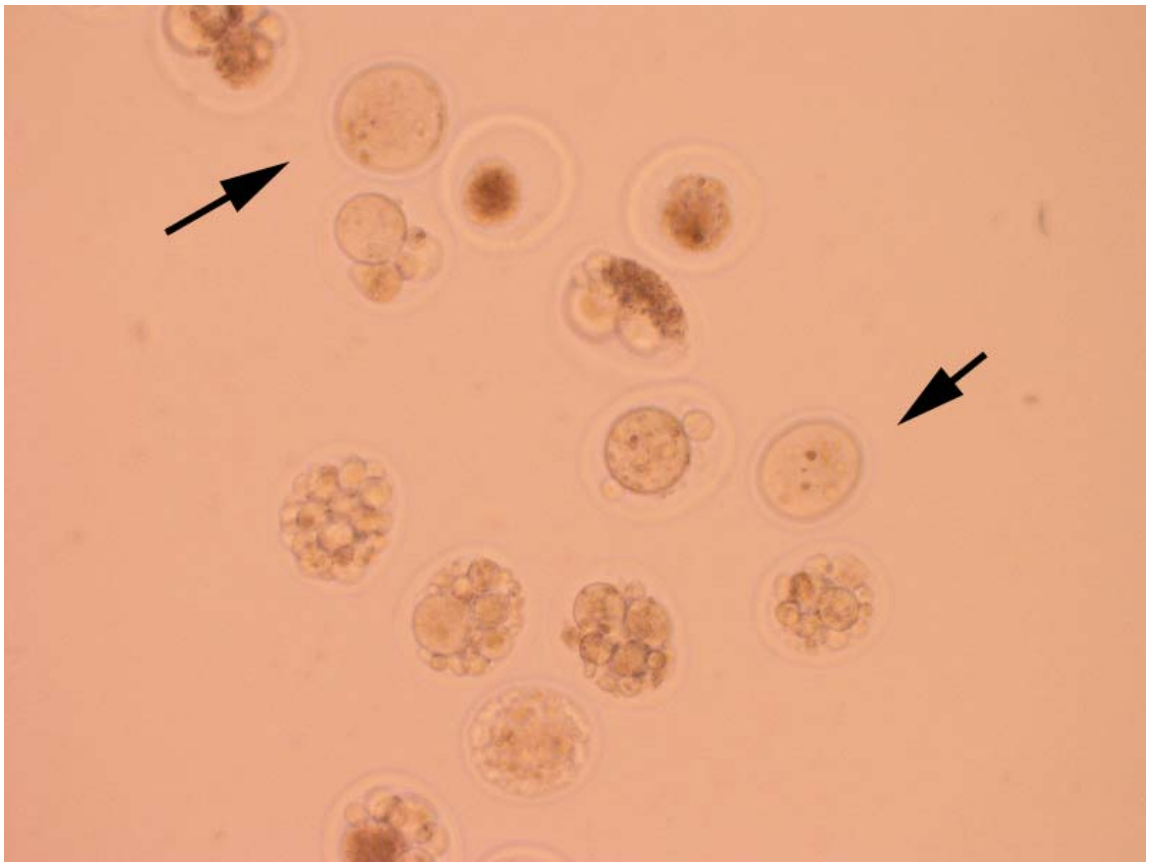

Figure 2. Embryos, Grade 3 and 4. The arrows indicate unfertilized ova. The rest of the embryos are either degenerating or dead, and cannot be used for transfer.

The procedure differed only slightly for an oviduct transfer. Females were superovulated and mated according to the same protocol mentioned before. The embryos were collected at 2.5 days post coitus, while at the 8-cell stage. Females were sacrificed via cervical dislocation and opened to harvest the oviducts and part of the uterine horns. The embryos were flushed into a dish with M2 solution by inserting a filled 1cc syringe with a dulled 30G needle into the infundibulum.

2.4 CRYOPRESERVATION OF EMBRYOS

The Biocool unit was turned on to allow it to reach proper temperature at -6^{0}C, and the prepared glycerol cryoprotectant (10.96 ml glycerol (embryo tested, G-2025 SIGMA) per 100ml final volume M2) and the sucrose solution (1.71 gm sucrose (embryo tested, S-1888 SIGMA) per 50ml final volume M2) were removed from the refrigerator, and put on ice. These solutions were filter sterilized and aliquoted into a sterile petri dish. Media dishes with M2 solution were set up to hold the embryos until ready to be frozen. Straws were labeled according to cryopreservation protocol: Straw number in the top left-hand corner, date frozen in the top right-hand corner, the line being frozen in the middle, and number of embryos in the straw in the bottom left-hand corner, along with the initials of the person performing the preservation. This information was also recorded in the freezing log book. A cotton plug was pushed down into the straw, and the measurements were marked (1cm, 7cm) on the straws with a cryo pen. Using a Monoject 1cc syringe, the sucrose solution was sucked up to the 7cm mark, followed by a column of air, then the glycerol solution to the 1cm mark, then a final column of air. Around 20-60 embryos were transferred into each straw in the glycerol section of the straw using a mouth pipet, followed by an air bubble and a small amount of sucrose. Both ends of the straw were heat sealed. Straws ready to be frozen were placed into the Biocool at -6^{0}C and left for 5 minutes. Afterward, straws were seeded using forceps dipped in liquid nitrogen. Once all straws were seeded, the freezing program was run on the Biocool. After the program was completed at an

ending temperature of approximately -46^{0}C, the straws were placed in liquid nitrogen for storage.

2.5 THAWING PROCEDURE FOR CRYOPRESERVED EMBRYOS

The straw was first removed from the liquid nitrogen tank and balanced horizontally between two racks for three minutes, making sure that the embryo column was not touching any surface. If present, condensation was wiped off with a Kim wipe before proceeding. The straw was held by the cotton tip and shaken like a thermometer until the air bubble rose to the top of the straw. It was then placed in the incubator at 37^{0}C in a water-filled beaker for three minutes. The straw was removed from the incubator and placed upside down in a second beaker filled with room-temperature water for three minutes. The end opposite the cotton plug was cut, then a cut made through the center of the cotton plug. A metal rod was used to push all the fluid from the straw into a petri dish, taking care that the cotton plug did not come out or touch the bubble of fluid. The embryos were then washed through several drops of M2 media and either cultured for later use or transferred to a recipient female immediately.

2.6 CULTURING PROCEDURE

To culture the embryos, M16 media was covered in embryo tested mineral oil and placed in a CO_2 water-jacketed incubator (5.7% CO_2, 37^{0}C). It was left in the incubator for several hours, usually overnight, to allow it to equilibrate before the embryos were placed in the media. 2.5 day embryos, anywhere from two- to eight-cells, were either used immediately in an oviduct transfer, or allowed to culture until they developed into blastocysts, usually after 24 hours. Embryos collected at 3.5 days, at

blastocyst stage, were used immediately in uterine transfers. Embryos collected at 0.5 days, at 1-cell stage, were mixed with hyaluronidase (2900 μL M2 plus 100 μL hyaluronidase) (10mg/ml) approx 2 minutes to remove cumulus cells, washed in M2 and then cultured to later stage embryos.

2.7 SURGICAL PROCEDURE

The recipient and control females were weighed and analgesic and anesthetic doses calculated. The anesthetic, a ketamine/xylazine cocktail, was given at 10mg/kg. The cocktail was mixed at 70mg/kg : 7mg/kg ketamine to xylazine. The ketoprofen analgesic was dosed at 10mg/kg and was mixed as 1mg/ml (0.1ml ketoprofen at 100mg/ml plus 9.9ml sterile water). Buprenorphine was dosed at 1mg/kg and was mixed as 1ml (0.3mg) buprenorphine plus 4ml sterile water. One hour prior to surgery, the buprenorphine (surgical and non) and saline mice were injected with appropriate doses of their respective analgesic. Ketoprofen mice were injected with their analgesic mixed with 2ml of warmed saline post-surgically. All mice were anesthetized with a ketamine/xylazine cocktail injected i.p. Mice were placed in a warmed standard mouse cage for five minutes post-injection to ensure they were asleep. Depth of anesthesia was determined by pinching toes and tail with a pair of forceps. The tail was tested first, and if no tail flick reaction occurred a toe on a hind paw was pinched. If a withdrawal reflex occurred, the depth of anesthesia was inadequate. Once appropriate anesthesia depth had been reached, the dorsal surgical area – from the last rib to 1 cm cranial to the base of the tail - was shaved and scrubbed with a 10% betadine solution and 70% isopropyl alcohol, and an artificial tear solution (Artificial Tears Ointment, Lubricant Eye

Ointment (sterile), Phoenix Pharmaceutical, Inc.) was placed on the eyes. To prevent anesthetic hypothermia, since an anesthetized mouse cannot regulate its body temperature, the animal was placed in a plastic petri dish lid set on top of a covered warmed slide warmer. Time was recorded at first incision.

In the uterine transfer procedure, a horizontal incision approximately 1 cm in length was made on the dorsal surface of an anesthetized, recumbent mouse. Blunt dissection was used to separate the skin from the fascia underneath. The incision was then positioned over the left ovarian fat pat, which can be seen through the transparent body wall. A small incision was then made to enter the body wall and the ovarian fat pad was exteriorized. A sterile bulldog clip was used to anchor the fat pad on the dorsum. The mouse was then placed under a dissecting microscope. A hole was pierced into the uterine wall with a 27G needle and the tip of a pulled glass pipette loaded with embryos was threaded through the hole into the uterus. Five or six embryos were blown into the uterine horn, along with a small amount of M16 solution. The pipette was checked to be sure all embryos had been transferred. The exposed uterine horn was moistened with a small amount of warmed saline and then returned to the body cavity. The body wall incision was closed with a single suture of 5-0 silk (Oasis; 5-0 Silk-oasis nonabsorbable suture; MV-682). The entire procedure was repeated for the right side with 10 or 12 embryos total per mouse being transferred.

In an oviduct transfer, an incision was made through the body wall avoiding nerves and large blood vessels. The incision was manipulated until the white fat pad surrounding the ovaries was visible. Dull forceps were used to grasp the fat pad and

gently pull it out through the incision. Using a microscope, the ovary was positioned for easy access to the oviduct then clamped outside the body with a small, sterile bulldog clip on the fat pad. Twelve embryos at 2.5 days (two- to eight-cell stage) were pipetted into a small drop of warmed M2 solution. A transfer pipette, with a tip diameter slightly larger than the embryos, was loaded with M2, followed by a 2-5 mm air bubble, and then the media with the 12 embryos for transfer. The pipette was then carefully set aside until it was time to use it. At this point, the mouse was moved back under the microscope. The bursa surrounding the oviduct was gently torn open with two pairs of forceps, giving the surgeon access to the infundibulum, the funnel-like dilation at the distal end of the uterine tube that provides a path from the ovary to the uterine horn. Tearing the bursa sometimes caused a small amount of bleeding, which was gently blotted up using a sterile cotton swab. The tip of the pipette loaded with 12 embryos was slid into the infundibulum and the contents gently expelled until the air bubble was visible within the oviduct. The pipette tip was carefully removed and checked beneath the microscope to ensure the embryos were transferred. The ovarian fat pad and uterus were gently replaced into the abdominal cavity, and the body wall closed with one or two interrupted sutures of 5-0 silk. Oviduct transfers were done on one side of the body only, while uterine transfers were done on both sides of the body. Embryos transferred into the infundibulum of the oviduct will migrate across both horns and distribute evenly in the uterus. Transferring an equal number of embryos into both horns, therefore, helps prevent dystocia due to a lack of equilibrium between the uterine horns.

In both procedures, the skin incision was closed with a sterile steel surgical clip and the surgical site scrubbed with hydrogen peroxide (Swan solution of Hydrogen Peroxide; 3% H_2O_2 U.S.P.) to clean it. The mouse was given 2ml of warmed saline – 1ml subcutaneously and 1ml i.p. – then placed on a water circulating heating pad. Mice in the ketoprofen group, surgical and non, were given their dose of ketoprofen mixed with the warmed saline. Each mouse was observed during the recovery period and times were recorded for first movement and walking. Upon demonstrating reliable movement, the mouse was returned to the warmed cage. Mice were identified via conventional ear marks in the right ear numbering 1 through 5.

2.8 OBSERVATIONS

Mice were left in the warmed cage for 4-5 hours before being placed in individual cages for observation. It was unknown to the observer which mouse was in which cage. Two observation periods of 5 minutes each were used, one at 1800 and one at 1830 hours. The mice were scored on three different parameters: Physical Condition, Behavior, and Posture (Table 1).

Physical Condition focused on observations on Hair Coat and Eyes and Nose, with a score from 0 (normal) to 2 (abnormal) being assigned. Behavior focused on Activity and Temperament, with 0 being normal and 3 being agitated and aggressive. Posture focused on Stance and Locomotion, with 0 being normal and 3 being prone and motionless.

Scores given were determined by the observer's subjective criteria, based on her observations of "normal" mice and of the surgical mice during recovery. Mice received a score of 1 in Hair Coat if they were not actively grooming during the observation, as this rating was confounded by the surgical preparation. For Behavior, if the mouse was not moving except for slight twitching it received a score of 1 in Activity. If it was moving around the cage, but at a fast pace punctuated by frantic digging or climbing the cage walls, the Activity was recorded as 0 but Temperament was recorded as a 1 or 2. For Locomotion, if the mouse was moving on its own it was recorded as 0. Movement following a single tap on the cage was recorded as 1. Several taps was recorded as 2, and if the cage had to be opened to produce movement it was recorded as 3.

Table 1. Behavior Scores. Scoring criteria adapted from Gillingham, et.al. (11)

Physical Condition		
Score	*Hair Coat*	*Eyes and Nose*
0	Normal, well-groomed	Normal, no discharge
1	Rough, dirty, ungroomed	Eyes closed or squinted, no discharge
2	Rough, hair loss, ungroomed	Eyes closed or squinted, discharge

Behavior		
Score	*Activity*	*Temperament*
0	Normal	Normal – not agitated
1	Not moving around	Mildly agitated
2	Agitated – chewing on cage	Moderately agitated
3	Very agitated, chews on feet/tail	Severe agitation, aggressive

Posture		
Score	*Stance*	*Locomotion*
0	Sitting in normal resting position	Voluntary locomotion
1	Sitting in hunched position	Slight stimulation needed
2	Hunched posture/head on floor	Moderate stimulation needed
3	Lying prone on floor	No locomotion after moderate stimulation

After the second observation, it was determined which mouse had received what treatment and scores were totaled for each period. The three surgical mice were housed together and the two non-surgical controls were euthanized. The three surgical females were observed daily for signs of pregnancy and separated into individual cages 5 days before their due date which was 18 days post-op. Females who did not appear pregnant during these 18 days were sacrificed on their due date and their uteri examined for signs of resorbed embryos. Females who did appear pregnant but did not give birth by 2 days past their due date were sacrificed and their uteri checked for signs of pregnancy. The date and number of pups born were recorded. The pups were checked daily and any change in numbers was recorded. Pups were weaned at 21 days and set aside for later use in the project.

2.9 STATISTICAL ANALYSES

An analysis of covariance (ANCOVA) was selected as the method to test our hypotheses. To test all of our null hypotheses that the means of one treatment group matched the means of another treatment group using the Students' *t* test would result in a grossly unacceptable Type I error, due to the fact that several tests would have to be run. In an analysis of variance (ANOVA), means of the variables of interest are compared by splitting the overall observed variance into different parts. This allows several hypotheses to be tested at once without increasing the probability of a Type I error. The statistical model of an ANCOVA follows the same model as an ANOVA, allowing multiple hypotheses to be tested at the same time without increasing the probability of Type I error.

Although our recipient females were identical in terms of strain and housing and were age-matched as closely as possible, their weights varied from under 20 grams to nearly 40 grams (Figure 3). In order to control for any effect this weight difference may have on our dependent variables, weight of the animal was set as a covariate. A *covariate* is defined as a variable that is possibly predictive of the dependent variables under study. Covariates can either be a confounder or an effect modifier.

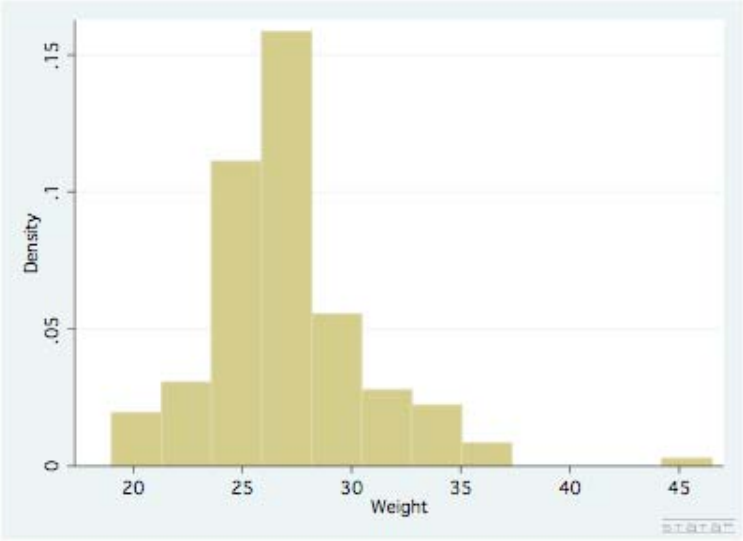

Figure 3. Weights of all Recipient Females. Histogram showing the distribution of weights for the recipient females. All three treatment groups (saline, ketoprofen, buprenorphine) and both surgical groups (uterine and oviduct) are represented.

The hypotheses tested using the ANCOVA method of analysis were the effects of the perioperative administration of ketoprofen or buprenorphine on the number of pups born and surviving to weaning age when compared with a saline control. Analyses were run for the two different types of embryo transfer procedures: uterine and oviduct.

An ANOVA was used to determine whether the administration of the analgesics had any effect on post-operative behavior when compared with a surgical saline control and a non-surgical analgesic control. Total scores for the first and the second observational periods were used, as well as total scores for the three major behavior categories.

Pearson's chi-squared test was used to analyze the relationship between the independent variables and a dichotomous outcome; namely, whether a recipient female had a litter or not. Chi-squared tests determine if the relative frequencies of the occurrence of observed events follow a frequency distribution; in other words, it tests for associations between factors. This allowed us to examine the extent to which exposure to one of our independent variables determined whether our females gave birth to a litter. Tests were run with the independent variables as given no dose or dose, where saline was compared with ketoprofen in one analysis and then with buprenorphine in a second analysis. In each case, the response variable of no litter/litter was examined.

The statistical program Stata was used to run our ANOVA/ANCOVA and chi-squared models.

3. RESULTS

3.1 UTERINE TRANSFERS

38 experimental groups underwent the uterine transfer procedure, each group consisting of three surgical mice and two non-surgical controls. The effects of administering ketoprofen, buprenorphine, and a saline control were evaluated in regards to number of pups born, number of pups weaned, as well as whether a litter was delivered or not. The number of pups born was a total count of all pups living or dead. The number of pups weaned chronicled only the animals that survived to weaning age (21 days). The weight of the recipient animal was taken into consideration as a covariate in an analysis of variance (ANOVA).

The number of litters born in the saline and ketoprofen groups is summarized in Table 2. Table 3 looks at the number of litters born in the saline and buprenorphine groups. Figure 4 depicts graphically the number of pups born and weaned in each of the treatment groups. Figure 5 shows the average number of pups born and weaned and the standard deviations across the three treatment groups.

Table 2. Number of Litters Born by Saline and Ketoprofen Groups, Uterine Surgery Only.

UTERINE	*Treatment Group*	
Litter Born	*Saline*	*Ketoprofen*
Yes	14	15
No	23	23

Table 3. Number of Litters Born by Saline and Buprenorphine Groups, Uterine Surgery Only.

UTERINE	*Treatment Group*	
Litter Born	*Saline*	*Buprenorphine*
Yes	14	17
No	23	20

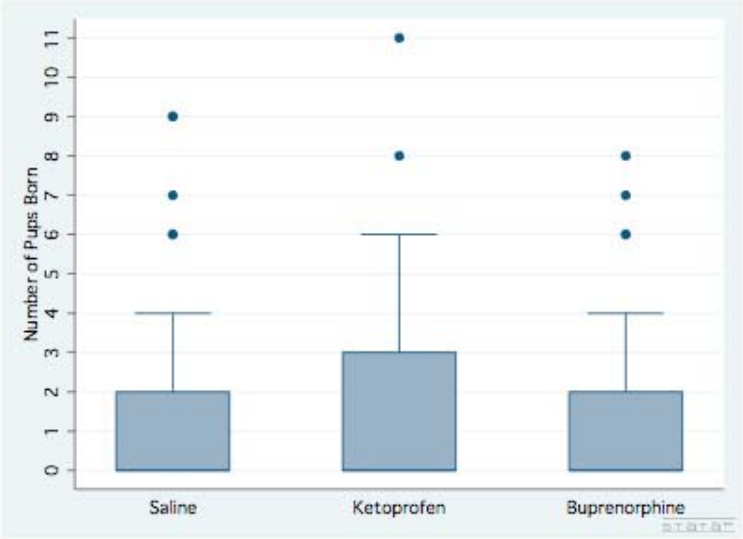

Figure 4. Number of Pups Born by Treatment Groups, Uterine Surgery Only. The median value in all three groups was 0. The average number of pups born to the saline group was 1.54, for ketoprofen the average was 1.60, and the average number for buprenorphine was 1.51.

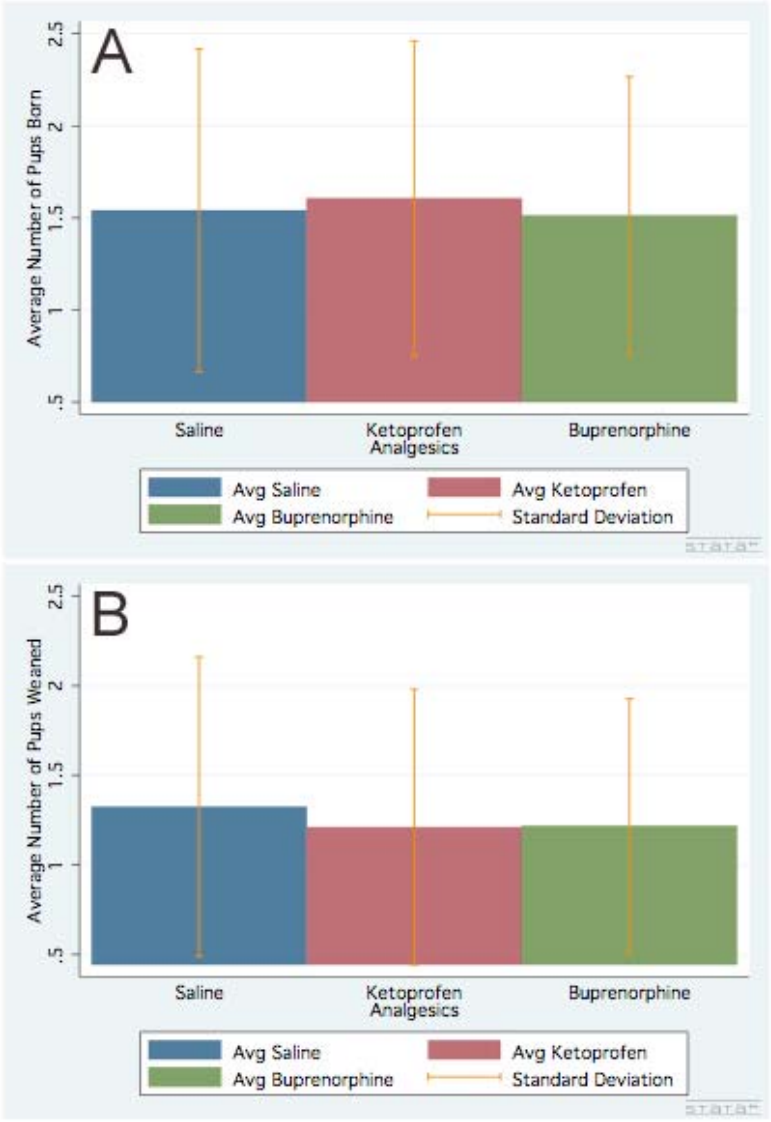

Figure 5. Summary of Pups Born and Weaned by Treatment Groups, Uterine Surgery Only. Figure 5A shows the average number of pups born across the three surgical treatment groups and the standard deviation for each group. There is no significant difference across the three groups at the 0.05 significance level. Figure 5B shows the average number of pups weaned across the three surgical treatment groups

(Figure 5, Continued) and the standard deviation for each group. Again, no significant difference is seen in the three groups at the 0.05 significance level.

An ANOVA table was used to determine any significant differences in number of pups born in the three treatment groups, using weight of the recipient animal as a covariate. The results from this analysis indicate that the number of pups born is not affected by the pre-operative administration of buprenorphine or post-operative administration of ketoprofen when compared with the administration of a saline control (P=0.9810).

The same analysis was used to determine any significant differences in number of pups weaned in the three treatment groups, once again using the recipient weight at a covariate. Once again, the results indicate that the administration of buprenorphine or ketoprofen, when compared with the administration of a saline control, has no effect on the number of pups surviving until weaning age (ANCOVA, P=0.9526).

Pearson's chi-square was used to determine any differences in numbers of litters born across the three treatment groups. Litters born was set as a "yes/no" response variable that did not take into account number of pups born or number of pups surviving until weaning. Two chi-squared tests were run, one that compared the saline treatment group to the ketoprofen group, and a second analysis comparing the saline treatment group to the buprenorphine treatment group. In this fashion, the outcome was evaluated on a "no dose/dose" basis. Analysis showed that there was no difference between the "no dose" treatment (saline) and the "dose" treatment (ketoprofen or buprenorphine) in

regards to whether a litter was born or not (P=0.884 with chi^2=0.02 for saline and ketoprofen, and P=0.4794 with chi^2=0.50 for saline and buprenorphine).

3.2 OVIDUCT TRANSFERS

15 experimental groups underwent the oviduct transfer procedure, each group consisting of three surgical mice and two non-surgical controls. The effects of administering ketoprofen, buprenorphine, and a saline control were evaluated in regards to number of pups born, number of pups weaned, as well as whether a litter was delivered or not. The number of pups born is a total count of all pups living or dead. The number of pups weaned chronicles only the animals that survived to weaning age (21 days). The weight of the recipient animal was taken into consideration as a covariate in an analysis of variance (ANOVA).

The number of litters born in the saline and ketoprofen groups is summarized in Table 4. Table 5 summarizes the number of litters born in the saline and buprenorphine group. Figure 6 depicts graphically the number of pups born and weaned in each of the treatment groups. Figure 7 shows the average number of pups born and weaned and the standard deviations across the three treatment groups.

Table 4. Number of Litters Born by Saline and Ketoprofen Groups, Oviduct Surgery Only.

OVIDUCT	*Treatment Group*	
Litter Born	*Saline*	*Ketoprofen*
Yes	8	7
No	3	3

Table 5. Number of Litters Born by Saline and Buprenorphine Groups, Oviduct Surgery Only.

OVIDUCT	*Treatment Group*	
Litter Born	*Saline*	*Buprenorphine*
Yes	8	7
No	3	4

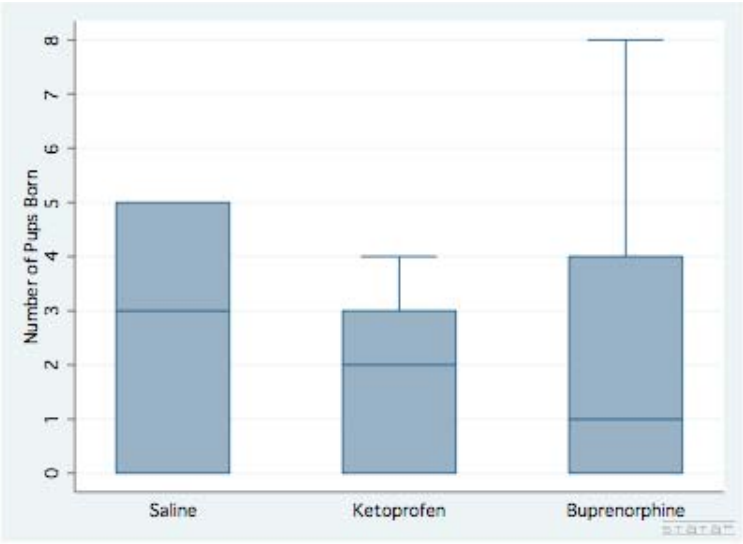

Figure 6. Number of Pups Born by Treatment Groups, Oviduct Surgery Only. The average number of pups born in the saline group was 1.55, for ketoprofen the average was 1.9, and the average number for buprenorphine was 2.

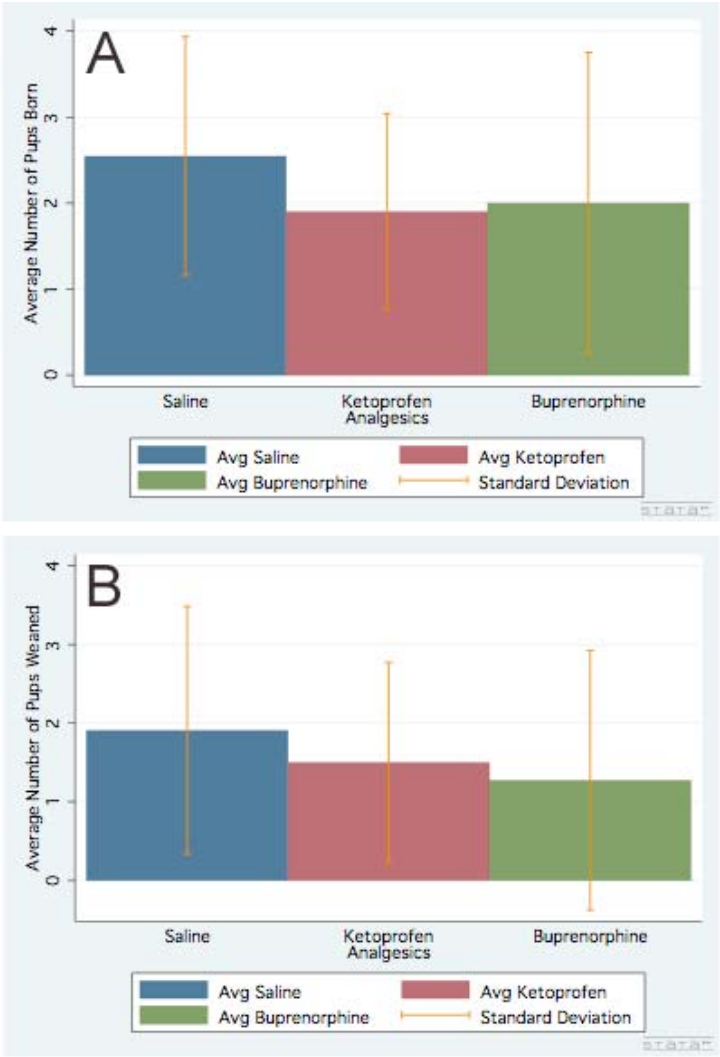

Figure 7. Summary of Pups Born and Weaned by Treatment Groups, Oviduct Surgery Only. Figure 7A shows the average number of pups born across the three surgical treatment groups and the standard deviation for each group. There is no significant difference across the three groups at the 0.05 significance level. Figure 7B shows the average number of pups weaned across the three surgical treatment groups and the standard deviation for each group. Again, no significant difference is seen in the three groups at the 0.05 significance level.

An ANOVA table was used to determine any significant differences in number of pups born in the three treatment groups, using weight of the recipient animal as a covariate. The results from this analysis indicate that the number of pups born is not affected by the pre-operative administration of buprenorphine or post-operative administration of ketoprofen when compared with the administration of a saline control (P=0.6876).

The same analysis was used to determine any significant differences in number of pups weaned in the three treatment groups, once again using the recipient weight at a covariate. Once again, the results indicate that the administration of buprenorphine or ketoprofen, when compared with the administration of a saline control, has no effect on the number of pups surviving until weaning age (ANOVA, P=0.6697).

Pearson's chi-square was used to determine any differences in numbers of litters born across the three treatment groups. Litters born was set as a "yes/no" response variable that did not take into account number of pups born or number of pups surviving until weaning. Two chi-squared tests were run, one that compared the saline treatment group to the ketoprofen group, and a second analysis comparing the saline treatment group to the buprenorphine treatment group. In this fashion, the outcome was evaluated on a "no dose/dose" basis. Analysis showed that there was no difference between the "no dose" treatment (saline) and the "dose" treatment (ketoprofen or buprenorphine) in regards to whether a litter was born or not (P=0.890 with a chi^2=0.02 for saline and ketoprofen, and P=0.648 with a chi^2=0.21 for saline and buprenorphine).

Several possible correlations beyond the original intent of this project were also tested. It was theorized at one point that the weight of the recipient animal may have an effect on whether she is able to carry the embryos to term and perhaps even how many she can carry to term. However, no correlation between weight and whether or not an animal had pups (P=0.1399), or between weight and number born (P=0.2332) was found.

This project utilized both fresh and frozen embryos, realizing that researchers engaging in embryo transfer operations would be using both types. For our purposes, "fresh" embryos included both those collected on the day of surgery and transferred immediately, as well as embryos collected a day early and allowed to incubate overnight. Possible correlations between embryo type (fresh or frozen) and whether or not a litter was born, as well as the number of pups born, were explored. Results showed that the type of embryo did not have any effect on litters born (P=0.9260) or number of pups born (P=0.2466).

3.3 BEHAVIOR

53 groups of mice, for a total of 265 individual animals, were studied over the course of this experiment. Mice were scored as individuals in their surgical groups and the results tabulated. Scores for each behavior category were compared between the surgical treatment groups (Saline, Ketoprofen, Buprenorphine), and the surgical versus nonsurgical control counterpart (Ketoprofen and Buprenorphine).

Although the behaviors enumerated in the scoring system were known as well-established indicators of discomfort in mice, the severity of discomfort based on those

behaviors is very subjective and controversial. Because of this subjectivity, the difference between a behavior score of 1 and 2 was not equal to the difference between 2 and 3.

To remedy this, behavior scores were simplified to a “normal” and “abnormal” response. The behavior criteria mentioned before were still used as a guideline, but now any abnormal behavior was recorded as a response regardless of severity.

For statistical purposes, the scores were further combined. A total score was taken for each observation period across the six individual behavior subcategories (hair coat, eyes and nose, activity, temperament, stance, and locomotion). In addition, the six subcategories were collapsed into three major categories: Physical condition consisted of hair coat and eyes and nose; behavior was activity and temperament combined; and posture took into account stance and locomotion. These scores were independent from and not included within the total scores for the observational periods.

Using an ANOVA table, the total behavior scores for periods one and two were analyzed by treatment groups, looking only at the mice that underwent surgery. Results indicated that the use of ketoprofen or buprenorphine had no effect on post-operative behavior when compared to a saline control ($P=0.8422$ for period 1 and $P=0.8307$ for period 2). Next, total behavior scores for the two periods were compared between the surgical mice who received ketoprofen and their non-surgical counterpart. Results indicated that surgery had no effect on the behavior of the mice who received ketoprofen ($P=0.8972$ for period one and $P=0.1607$ for period two). Finally, total behavior scores for the two periods were compared between the surgical mice who received

buprenorphine and their non-surgical counterpart. Surgery did not have any effect on the behavior of the mice who received buprenorphine (P=0.1636 for period one and P=0.2761 for period two).

Behavior scores for the three major categories were evaluated next, using an ANOVA table. The mice that received surgery were assessed across the treatment groups first. Physical condition scores for periods one and two were not affected by the administration of ketoprofen or buprenorphine when compared to a saline control (P=0.4845 and P=0.0973), and neither were the behavior scores (P=0.3651 and P=0.5326) or the posture scores (P=0.3515 and P=0.8745).

The mice that received ketoprofen, surgical and non-surgical control, were evaluated next. Surgery did not have an effect on physical condition scores (P=0.7589 for period 1 and P=0.6240 for period 2), the behavior score for period one (P=0.8516), or the posture scores (P=0.8700 for period 1 and P=0.1832 for period 2). However, surgery did have an effect in the behavior score for period two ($P < 0.05$; see Figure 8).

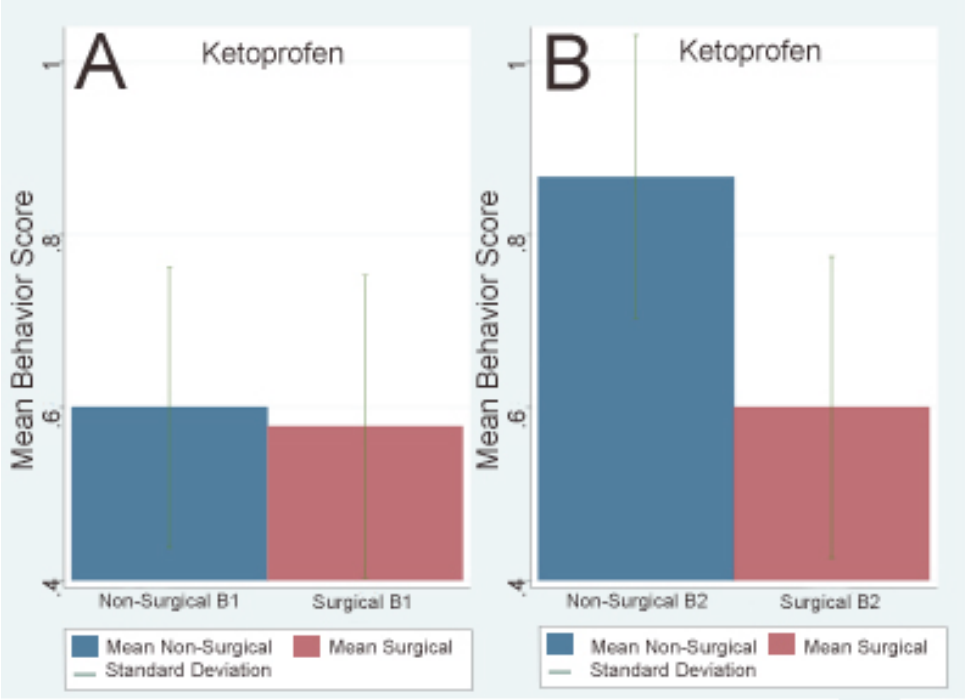

Figure 8. Summary of Behavior Scores by Period and Surgical Treatment, Ketoprofen Only. Bar graph depicting the average behavior scores and standard deviations for the mice receiving ketoprofen, surgical (red) and non-surgical (blue), for periods 1 and 2. Figure 8A shows the average scores for period 1 and no significant difference between the non-surgical control and surgical mice. In Figure 8B, there is a significant difference between the higher score of the non-surgical control and the surgical mice.

The mice that received buprenorphine, surgical and non-surgical control, were evaluated next. Surgery did have an effect on the physical condition score for period one ($P < 0.05$; see Figure 9). However, the physical condition score for period two ($P=0.1492$), behavior scores for periods one and two ($P=0.2802$ and $P=0.7569$), and

posture scores for periods one and two (P=0.8879 and P=0.2589) did not show to be affected by presence or absence of surgery.

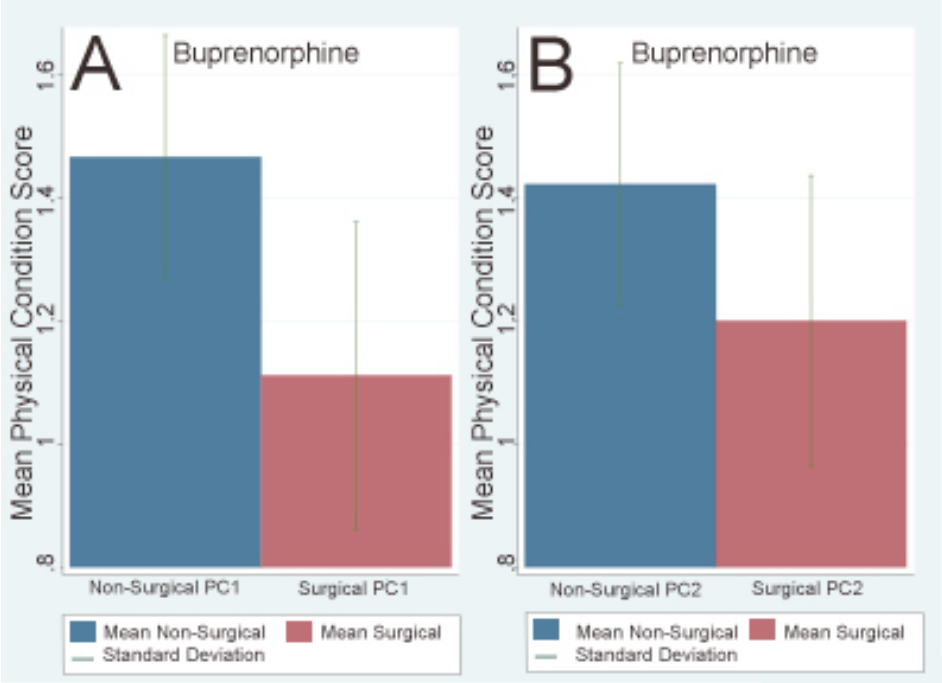

Figure 9. Summary of Physical Condition Scores by Period and Surgical Treatment, Buprenorphine Only. Bar graph depicting the average physical condition scores and standard deviations for the mice receiving buprenorphine, surgical (red) and non-surgical (blue), for periods 1 and 2. Figure 9A shows the average scores for period 1 and a significant difference between the higher score of the non-surgical control and the surgical mice. In Figure 9B, there is no significant difference between the non-surgical control and the surgical mice for period 2.

One of the motivations behind this project was the theory that analgesics might relieve some of the post-operative stress on the mice and thereby allow for greater

numbers of embryos to implant and develop to term. To test this theory, correlations between the post-operative behavior scores and whether or not a litter was born, as well as the number of pups born were evaluated.

Total behavior scores for periods one and two were tested against whether a litter was born with no significant results (P=0.6016 and P=0.4314), indicating that the behavior scale used in this experiment did not predict whether a mouse would carry a litter to term. The total behavior scores were run again, this time against the number of pups born. Again, however, there was no significant difference (P=0.2196 and P=0.4063), indicating that the behavior scale used did not predict how many pups would be born.

Scores for the major behavior categories were tested against whether a litter was born or not; however, none of the tests yielded significant results (P=0.6279 and P=0.2714 for physical condition; P=1.0000 and P=0.8877 for behavior; P=0.4706 and P=0.3314 for posture), once again showing that the behavior scale used did not predict whether a mouse would produce a litter.

Finally, scores for the major behavior categories were tested against the number of pups born, once again with no significant results (P=0.3418 and P=0.7493 for physical condition; P=0.4213 and P=0.5037 for behavior; P=0.7875 and P=0.6165 for posture), showing that the behavior scale used did not predict the number of pups born.

4. CONCLUSIONS

The results from this study indicate that neither the NSAID-class drug ketoprofen nor the opioid-class drug buprenorphine have any significant effects on the number of mouse pups born or weaned, or the number of litters born, following embryo transfer procedures when compared to a saline control. These results are not affected by the type of transfer procedure, uterine or oviduct, nor the condition of the embryos themselves, i.e. fresh or cryopreserved.

Further analysis of the data indicates that, within a range of weights, there is no correlation between the weight of the recipient female and her ability to carry the embryos to term, either in the number of pups born or whether she gave birth to a litter or not. Results also indicate that the condition of the embryos (fresh or cryopreserved) do not have any bearing on the number of pups born or whether or not a litter was produced.

Post-operative behavior was affected only in two categories, and only between the non-surgical control mice and their surgical counterpart. The ketoprofen mice differed significantly in their behavior scores for the second observation period, and the buprenorphine mice differed significantly in their physical condition scores for the first observation period. It is possible that this difference is due to the fact that the non-surgical mice were not as stimulated as the surgical mice, and so felt the effects of the anesthetic longer and more intensely than the surgical mice. This phenomenon was remarked upon during the collection of the raw data; the observer mentioned that the non-surgical mice tended to be more lethargic and did not move around or groom

themselves as much as the surgical mice did. The non-surgical mice did not appear to be in any discomfort; they simply appeared tired.

Apart from these two, the remainder of the analyses did not show any significant effect either between the three surgical treatments or between the non-surgical animals and their surgical counterpart in any of the categories studied.

In the original, ground-breaking work in embryo transfers accomplished by McLaren and Mitchie, the effects of surgery in general and self-styled traumatic surgery specifically were studied. Their results indicated that the number of pups born to animals subjected to a surgical embryo transfer was not statistically significant when compared with non-surgical animals pregnant by natural means. However, when subjected to traumatic surgical procedures, the rate of resorption of the transferred embryos increased dramatically when compared to animals receiving non-traumatic surgical procedures.

Although the type of surgery in that historic experiment was controlled for, sometimes there are problems that the investigator or surgeon cannot anticipate that result in a traumatic experience for the animal. Use of analgesics in surgical procedures could forestall the discomfort and pain to the animal brought about by unforseen problems.

One of the more recent developments arising from the surgical technique pioneered by McLaren and Mitchie is the use of cryopreservation and embryo transfer to preserve and then reconstitute a genetic line. Our experiment utilized both fresh and cryopreserved embryos with the two different surgical types. Neither ketoprofen nor

buprenorphine had any noticeable effects on either embryo type. Researchers who were reluctant to introduce another variable into an already complicated procedure such as reconstituting a preserved genetic line may wish to reconsider this position. Using an analgesic, while not producing any significant increase in transfer efficiency, does not harm cryopreserved embryos.

Embryo transfer procedures are already very widespread and the number of people using these techniques on a daily basis is increasing rapidly, especially since it is now possible for researchers to create a genetic line of their own experimental design through gene targeting. Embryo transfer is of particular import in this endeavor, since it is through this procedure that manipulated embryos are placed into the uterus of a recipient female and carried to term. Without this surgical manipulation, the work gone into creating a transgenic or targeted mutant mouse stops at the embryonic level.

The study undertaken here did not include the possible effects of ketoprofen or buprenorphine on manipulated embryos of any kind, either transgenic embryos where purified DNA is injected into the pronucleus of a zygote, or targeted mutant embryos where ES cells with a DNA construct are injected into the donated blastocysts of superovulated mice. Designing an experiment to examine such effects is highly recommended as a follow-up study, due to the increasing prevalence of such techniques and technology in laboratories today.

The selection of mouse strains in genetic manipulation procedures is critical; the eggs must be hardy enough to survive the manipulation procedure. The embryo strain tested in this study, C57BL/6, was used as the donor strain because it is the most

common background strain employed when creating targeted mutant embryos, shown time and again to be one of the most hardy embryonic strains. Unmanipulated embryos of this strain did not react adversely to the presence of ketoprofen or buprenorphine; however, testing of manipulated BL/6 embryos is encouraged as a follow-up study.

Other strains used in genetic manipulation procedures should be tested as well, first using unmanipulated embryos and then manipulated ones. While C57BL/6 embryos of 8-cell and blastocyst stages did not exhibit adverse effects with the introduction of ketoprofen or buprenorphine into the recipient mother, embryos of other strains might not fare as well.

Animals under stress can give confounding or unusable responses in experimental procedures. Stress can come from a variety of sources, such as room environment, the scent of blood or animals in estrus or other noxious stimuli, pain, and experimental manipulation, to name a few. Some of these stresses we cannot control for, but through the use of analgesics we can help ameliorate discomfort or pain in an experimental animal, and thereby decrease the amount of stress affecting it.

Opioids and NSAIDs were the two analgesic classes chosen for this experiment due to their actions at providing analgesia as well as their popularity in veterinary medicine. NSAIDs are considered the most popular class of analgesics in use in small animal veterinary medicine. Despite this popularity, however, neither drug type has been extensively studied for use in small rodents. Very few clinical trials have been used to determine the effect of buprenorphine or ketoprofen on small rodents. Paul

Flecknell has done tremendous work with these drugs and other analgesics in the rat, but the mouse has been sorely neglected (9).

In our work, we administered ketoprofen mixed with warmed saline immediately post-operatively, while buprenorphine was used one hour pre-operatively in order to give it time to reach its full analgesic potency. Using the drugs in this way, our results show that they do not cause any adverse effects on the mouse in terms of physiology and behavior.

Many researchers are reluctant to use analgesics on experimental animals for multiple reasons. The most common reason cited, and the one that led directly to this study, was the observation that older studies did not utilize analgesics. They are concerned that the addition of an extra variable may confound or complicate experimental results and render their data null and void. The work done in this study indicates that this is not a problem. No differences were found in either the use of the NSAID ketoprofen or the opioid buprenorphine in our results when compared to a saline control.

This study was designed to look at the effects of NSAID-class and opioid-class analgesics on embryo transfer procedures, specifically the effects on efficiency of transfer. Although our results indicate there is no difference in transfer efficiency whether using an analgesic or not, further and more specific studies focusing on one of the many topics studied here are recommended. Additional data on oviduct procedures, especially the effect of these analgesics on earlier-stage embryos, is an area of further recommended study. Though the study of the effects of analgesics on post-operative

behavior was secondary to the larger picture of the effects on embryo transfer efficiency, behavior-specific studies are also recommended, with a more expanded and detailed method of determining behavior scores.

In conclusion, although further studies are recommended, based on our results we will continue to use analgesics in our transfer procedures. Because analgesics do not appear to have any adverse effects on transfer efficiency rates, the adaptation of existing embryo transfer protocols to incorporate these drugs is a positive advance in animal health and comfort in comparative medicine.

REFERENCES

1. **Betteridge KJ**. 2003. A history of farm animal embryo transfer and some associated techniques. Anim Reprod Sci **79**:203-244.

2. **Betteridge KJ**. 1995. Phylogeny, ontogeny and embryo transfer. Theriogenology **44**:1061-1098.

3. **Capecchi MR**. March 1989. The new mouse genetics: altering the genome by gene targeting. Reviews; TIG **5**(3).

4. **Chin H, Wang C**. 2001. Uteral-tubal transfer of mouse embryos. Genesis **30**:77 81.

5. **Crosignani PG, Rubin BL** (Ed). 1983. Proceedings of the Serono Clinical Colloquia on Reproduction 4: *in vitro* fertilization and embryo transfer. London: Academic Press, Inc.

6. **Curtis JL**. 1990. Cattle embryo transfer procedure. Burlington, Massachusetts: Academic Press, Inc.

7. **Didion, BA, Hauser ME, Eisen EJ**. 1991. Use of *in vitro* fertilization and embryo transfer to circumvent infertility caused by an inherited imperforate vagina in mice. J In Vitro Fert Embryo Transf **8**(3):167-171.

8. **Doehmer J**. April 1982. Introduction of rat growth hormone gene into mouse fibroblasts via a retroviral DNA vector: expression and regulation. Proc Natl Acad Sci U S A **79**(7):2268-2272.

9. **Flecknell P, Watermann-Pearson A**. 2000. Pain management in animals. London: W.B. Saunders.

10. **Gaynor JS, Muir W.** 2002. Handbook of veterinary pain management. St. Louis, Missouri: Mosby, Inc.

11. **Gillingham MB, Clark MD, Dahly EM, Krugner-Higby LA and Ney DM**. 2001. A comparison of two opioid analgesics for relief of visceral pain induced by intestinal resection in rats. Contemp Top Lab Anim Sci **40**:21-26.

12. **Hahn J**. 1984 The value of laboratory animal models in embryo transfer research. Theriogenology **21**(1):45-59.

13. **Hickman D, Thompson K.** 2004. Multi-phase approach to eradicate enzootic mouse coronavirus infection. Contemp Top Lab Anim Sci **43**(5):22-28.

14. **Hogan B, Beddington R, Constantini F, Lacy E.** 1994. Manipulating the mouse embryo, a laboratory manual. Second edition. New York: Cold Spring Harbor Laboratory Press.

15. **Jackson Laboratory** [Internet]. C57BL/6 strain information sheet. Available at http://jaxmice.jax.org/jaxmicecgi/jaxmicedb.cgi?objtype=pricedetail&stock=000664 Accessed on 10/15/05

16. **Johnson LW, Moffatt RJ, Bartol FF, Pinkert CA**. 1996. Optimization of embryo transfer protocols for mice." Theriogenology **46**:1267-1276.

17. **Lauria A, Gandolfi F** (Ed). 1992. Embryonic development and manipulation in animal production. London: Portland Press.

18. **McLaren A, Michie D.** 1956. Studies on the transfer of fertilized mouse eggs to uterine foster-mothers. J Exp Biol **33**:394-416.

19. **McLaren A, Michie D.** 1959. Implantation and foetal mortality after induced superovulation in females of various ages. J Exp Biol **36**:281-300.

20. **Mochida K, Wakayama T, Takano K, Noguchi Y, Yamamoto Y, Suzuki O, Matsuda J, Ogura A.** 2005. Birth of offspring after transfer of mongolian gerbil (*Meriones unguiculatus*) embryos cryopreserved by vitrification. Mod Reprod Dev **70**:464-470.

21. **Nagy A, Gertsenstein M, Vintersten K, Gehringer R**. 2003. Manipulating the mouse embryo, a laboratory manual. Third edition. New York: Cold Spring Harbor Laboratory Press.

22. **National Research Council**. 1996. Guide for the care and use of laboratory animals. Washington, D.C.: National Academy Press.

23. **Okamoto M, Matsumoto T**. 1999. Production of germfree mice by embryo transfer. Exp Anim **48**(1):59-62.

24. **Post Graduate Committee in Veterinary Science, University of Sydney**. 1992. Embryo transfer and pregnancy diagnosis, proceedings 189. University of Sydney, Australia.

25. **Pullium J, Homberger F, Benhamin K, Dillehay D, Huerkamp M**. 2003. Confirmed persistent mouse hepatitis virus infection and transmission by mice with a targeted null mutation of tumor necrosis factor to sentinel mice, using short term exposure." Comp Med **53**(4):439-443.

26. **Sherwood L.** Human physiology: from cells to systems. Fourth edition. Pacific Grove, California: Brooks/Cole.

27. **Silver LM** [Internet]. 1995. Mouse genetics: concepts and applications. Oxford University Press. Available from http://www.informatics.jax.org/silver/index.shtml

28. **University of Illinois at Chicago.** Winter 2001. Transgenic mouse technology. The Reporter **2**(4).

29. **UTMB Sealy Center for Cancer Biology** [Internet]. Transgenic mouse general information. Available from http://www.utmb.edu/scccb/mouse/default.asp Accessed on 10/15/05

30. **Vergara GJ, Irwin MH, Moffatt RJ, Pinkert CA**. 1997. *In vitro* fertilization in mice: strain differences in response to superovulation protocols and effect of cumulus cell removal. Theriogenology **47**:1245-1252.

31. **Wayss K, Klefenz M, Schenkel J**. 2005. Cryopreservation of transgenic mouse embryos – an eight years' experience. J Exp Anim Sci **43**:69-85.

32. **Wolf DP, Quigley MM**. 1984. Human *in vitro* fertilization and embryo transfer. New York: Plenum Press.

VITA

Name:	Heather Ann Burckhardt
Address:	2903 Valley Manor Dr., Kingwood, TX, 77339
Email Address:	haburckhardt@yahoo.com
Education:	B.S., Biomedical Sciences; Texas A&M University, 2003 M.S., Laboratory Animal Medicine; Texas A&M University, 2006